

A NEW METHOD FOR STAINING
TUBERCLE BACILLI, APPLICABLE ALSO
TO THE MICRO-ORGANISM OF LEPROSY
AND OTHER ACID-FAST GERMS

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VILHELM HALLBERG

*This paper will also be published as Supplementum CLXXX
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By due permission of the most experienced
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FROM THE DEPARTMENT OF HYGIENE AND BACTERIOLOGY
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CHAPTER I.

A brief Survey of some Previous Methods.

Since the discovery by ROBERT KOCH, in 1882, of the causative agent of tuberculosis a great number of methods for its staining have seen the light of day.

Koch himself (23, 24, 25) used a solution of methylene-blue, which had been alkalified (distilled water 200 cc + 1 cc saturated solution of methylene-blue in concentrated alcohol + 0.2 cc of 10 per cent. potassium hydrate). He allowed this stain to act on smears and sections of tuberculous material during 20 to 24 hours at room-temperature. Later on, by the use of a staining solution of 40° C. (water-bath) Koch was able to reduce the time to $\frac{1}{2}$ to 1 hour. After that, the preparations, having passed through water, were kept in a saturated aqueous solution of vesuvin for some minutes, once more washed in water, and dried. On examination under the microscope the tubercle bacilli showed a slightly blue colour, while other bacteria were stained brown. Koch was of the opinion that vesuvin possessed the ability to force the methylene-blue out of the common microbes, but not off the tubercle bacilli.

Ehrlich (5), who had paid particular attention to the fact that the staining solution used by Koch was alkalified and, moreover, considered the presence of alkali indispensable in order to enable the stain to penetrate the cover of the tubercle bacilli, decided, in 1882, to try another alkali and other staining matters: Methyl-violet or fuchsin solved in concentrated and filtered aniline water. — With regard to the details of the preparation of the said stains, vide the author's original papers. — The slides containing tubercle bacilli were subjected to the Ehrlich stains for $\frac{1}{4}$ to $\frac{1}{2}$ hour at room-temperature¹ and then, for some few seconds each, first to strong nitric acid

¹ Since Rindfleisch (37), in 1883, had introduced the procedure of heating the slide more strongly than Koch did, the time was shortened.

and then to weak solutions of vesuvin and methylene-blue, respectively. The tubercle bacilli assumed a distinctly blue or red colour respectively, while other bacteria were stained yellowish-brown or blue.

Koch, who admitted the superiority of Ehrlich's method to his own, suggested the addition of 10 cc absolute alcohol to the anilised solution in order to maintain its efficiency somewhat longer (approximately 10 days). Moreover, Koch recommended that the treatment with acid should be followed by a further decolorization with 60 to 70 per cent. alcohol.

Ziehl (48), in 1882, rejected Ehrlich's assertion about the indispensability of alkalis to the stains. Thus, he proved that carbolic acid could be substituted for aniline. Tubercle bacilli, subjected to a solution consisting of distilled water in which had been dissolved the said acid and methyl-violet, were coloured as distinctly as when using the Ehrlich method. Moreover, the carbolic solution remained efficacious for quite a long time.

Neelsen (29), in 1883, used a slightly alcoholised 5 per cent. carbolic aqueous solution to which had been added $\frac{3}{4}$ per cent. fuchsin, and for decolorization 25 per cent. sulphuric acid. — No details are to be found in his paper.

This method, more perfected, is known under the name of Ziehl-Neelsen's: Staining with concentrated carbol-fuchsin, decolorization with a mixture of alcohol and hydrochloric acid, and counter-staining with methylene-blue. The tubercle bacilli appear to be red, while common microbes, as well as the background, display a blue colour.

Even if among all the other procedures for staining Koch's bacillus some are very good, the Ziehl-Neelsen method has maintained its superiority by reason of its simplicity and solidity.

CHAPTER II.

Origin of the Nachtblau Method for Staining Tubercle Bacilli and its Preliminary Use.

Freundlich and Neumann (6) classify organic staining matters in water solutions, in regard to their ability of diffusion etc., into the following three groups: A. Real solutions (crystalloids); B. Semi-

colloidal solutions; C. Colloidal solutions. On reading the enumeration of the different substances — rendered in a manual on chemistry by my teacher, the late professor S. G. Hedin (15), Upsala — I was struck by the fact that all of those which had been used for staining tubercle bacilli belonged to the first two groups.

When, several years later, I had become a collaborator in the research-work into the morphology of the micro-organism of tuberculosis, proceeding at the Institute of Hygiene and Bacteriology at Upsala, a small flask containing *Nachtblau* one day happened to fall into my hands. I then recollected the above fact that *Nachtblau* in aqueous solution behaved in the same way as a colloid, and decided to try if this substance might perhaps be useful for staining tubercle bacilli.

Thus, in June, 1939, I dissolved 0.5 g of *Nachtblau* (Grübler & Co.) in 10 cc 95 per cent. alcohol, and of this solution I added 1 cc to 10 cc carbolic (5 per cent.) distilled water. This stain was poured on to a film prepared from a Koch-bacillus culture and heated to vaporization three times. After this the slide was decolorised according to Ziehl-Neelsen and counter-stained with diluted carbol-fuchsin for a few seconds. On examination under the microscope it was found that the tubercle bacilli displayed a distinctly dark-blue colour, and appeared larger on the red back-ground. — With regard to more closely elaborated solutions of *Nachtblau*, counter-stains, decolorising fluids etc. vide Chapters IV and V.

The same procedure was then carried out on sputa from patients suffering from pulmonary tuberculosis, the result being that the tubercle bacilli appeared as just described and seemed to occur in greater numbers than in corresponding films stained by Ziehl-Neelsen's method, while the other bacteria of the buccal flora took the counter-stain, but with only one exception. It was thus possible to detect already in a couple of the first stained films, the same as later on from time to time, some cells (ovoid, round, and branched) of a yeast-oidium like fungus, the majority being counter-stained, but many of them had taken the same dark-blue colour as the tubercle bacilli, proving that both organisms contain a substance to which *Nachtblau* possesses the same affinity.

The said fungus corresponded fully to that which Reenstierna (34), in 1912, found in different tuberculous material (sputa, urethrocatheterized urines, etc.), and which he considers as a maternal fungus

of the tubercle bacillus by reason of its ability, (studied in *single-cell* culture) to produce acid-fast rods with the same morphological and tinctorial characteristics as Koch's bacillus, observations which have been confirmed and developed by Gullberg (11) and Hollström (18, 19, 20).

The majority of the fungus cells take the counter-stain in the same way as by use of the Ziehl-Neelsen method. It seems, however, that, with the use of Nachtblau, a larger number of them appear acido-resistant, resulting in the fact that the fungoid elements are more easily detected.

Further examinations of tuberculous sputa confirmed the aforesaid observations.

Films which originally have been stained according to Ziehl-Neelsen can easily be stained after with Nachtblau, but the immersion oil must first be removed by xylol, and the latter then allowed to evaporate properly.

The first results of the Nachtblau-staining (12) were laid before the Association of Swedish Pathologists at its meeting in Upsala on November 4th, 1939.

The method was then tried on films from other tuberculous material, and also on sections. The elaboration of the procedure had in 1941 reached a rather complete stage (13), whose rules, with some modifications, still hold good.

CHAPTER III.

Soluble Properties of Nachtblau, Stability of Solutions, and pH.

The carbolic Nachtblau solution of the composition described in my aforesaid paper of 1941, remained efficacious for at least one year. The material employed, originating from various flasks, was easily soluble in distilled water. I believed at that time that all the numbers issuing from the Grübler factory, were equivalent.

Then, in 1942, Roulet published a paper (38) in which, having confirmed my statements as to the staining of tubercle bacilli, and emphasizing the superiority of the Nachtblau method (vide Chapter

VI), he says that the staining solution does not keep efficacious longer than a few days.

Somewhat struck with this assertion, I thought that Roulet happened to deviate from my original description, e.g. that the stock solution had not been sufficiently concentrated or not well enough shaken; or that too much alkali had been added to the staining solution or some sediment, on preparing the latter, had accompanied it from the bottom of the stock solution. But the meticulous manner which could be traced throughout the paper by Roulet, convinced me that the difference in our solutions in respect of their remaining efficacious, must be due to something else. I then asked myself if the quality of the *Nachtblau* material used by Roulet might not have been inferior, and procured several flasks with *Nachtblau* of different makers' numbers, two of which were labelled "*Nachtblau, Botanik, Gramfbg*". I was then able to state that some of the flasks, among them the two with the said superscription, contained *Nachtblau* producing solutions which, when carbolic acid had been added, retained their efficacy for a great length of time, while solutions analogously prepared with *Nachtblau* from the other flasks were efficacious only for a short period. Furthermore, it was stated that *Nachtblau* dissolves in distilled water, but in different degrees for the superior and the inferior numbers of the material. Thus, if we sprinkle a few grains of the former sort on the surface of the water in a glass, they will dissolve rapidly on sinking to the bottom, and produce a characteristic blue rain, which then spontaneously, without the glass being moved, change into a homogeneous, dark-blue solution. The same process, carried out with the latter sort, produces the following effect: The particles of the substance remain for a while floating on the surface, whereupon they dissolve slowly and diffusely, resulting in a solution with a faded blue tint. It then became evident that it was the very staining solutions prepared from this material, which dissolves only slowly in water, which retained their efficacy for only a short period, as yet for about a couple of weeks according to the maker's number. On inquiring into the matter, the Grübler factory supplied the information that, chiefly on account of difficulties brought about by the war, they were no longer able to produce *Nachtblau* of the superior sort.

Apart from this difference in water solubility between *Nachtblau* of superior and inferior quality, differences have on examination been

stated inter alia regarding the pH and the percentage of Cl in the respective staining matters as is seen from the following:

Table

Distilled water cc	Liquid (90 %) phenol cc	Nachtblau sat. sol. in 95 % ethanol Superior quality cc	Inferior quality cc	Potassium hydroxide 10 % cc	pH
90	0	10		0	2.4
90	0		10	0	4.86
90	2.5	10		0	2.39
90	2.5		10	0	3.62
90	2.5	10		0.2	2.84
90	2.5		10	0.2	6.33

On analysis, Nachtblau of superior quality was found to contain 0.263 per cent. of chlorine, while dye of inferior quality contained 4.73 per cent.

The first condition for obtaining a stable carbohic acid stain is thus to make use of Nachtblau of sufficiently good quality.

Nachtblau dissolves rather poorly in a cold solution of carbohic acid, but well when heated to boiling. Such a staining solution alone can thus be used. But I prefer, for practical reasons, to prepare first a saturated alcoholic stock solution, and from this the staining solution. The presence of alcohol diminishes somewhat the colloidal state of the Nachtblau, but increases, on the other hand, the time during which the solution is efficacious. The carbohic acid can also previously be added to the stock solution after which one has only to add 10 cc of the same to 90 cc distilled water.

Regarding the concentration of the carbohic acid solution, it should be recollected that Neelsen dissolved fuchsin in one of 5 per cent. This concentration is also suitable in the case of Nachtblau. When making a titration of the indispensable quantity of the acid in question, I found that it was possible to go as low as 0.6 per cent. without any appreciable deterioration or diminishing of the staining capacity. On the other hand, when exceeding 5 per cent., the tubercle bacilli take the stain more weakly or faintly. As it was thus proved that 5 per cent. carbohic acid was unnecessary, I decided to use half the quantity. To the water in which the carbohic acid is to be dissolved,

I added subsequently potassium hydrate (maximum 0.2 cc of a 10 per cent. potassium hydrate solution to 100 cc distilled water). In such alkaliised water carbolic acid dissolves more easily than in water only. If, on the other hand, more than 0.2 cc of the said solution is added, the *Nachtblau*, being basic in itself, will be precipitated by the potassium hydrate.

I am under the impression that the tubercle bacilli appear more compactly stained (no gaps in the body of the bacillus) when potassium hydrate is added to the carbolic *Nachtblau* solution, a circumstance which in routine work may facilitate the detection of the bacilli. This, however, holds good only for the above sort of *Nachtblau*, which easily dissolves in distilled water. Alkali added to the sort of the matter which is difficult to dissolve causes rather damage, as the matter is precipitated.

The stock solutions appear to retain their efficacy for a practically unlimited length of time, even if only inferior *Nachtblau* is available. In respect of the staining solutions may be said: Those containing carbolic acid, and which are used for the *successive method* (vide infra), are efficacious for at least one year if superior matter is used, and, extremely capricious, for a few days up to some weeks, when inferior qualities are employed. In the former case dye deposits certainly occur on the bottom and walls of the flask, which gradually enfeeble the solution. It is, therefore, advisable not to use the same solution for any length of time. In the latter case the matter is soon precipitated, and this results in a fresh solution having to be frequently prepared, sometimes every day.

Attempts have therefore been made so that also the *Nachtblau* difficult soluble in distilled water gives stable solutions. A saturated alcoholic solution of this kind, dissolved in greatly diluted hydrochloric or nitric acid, stains the bacilli in question a faint blue, while, if dissolved in sulphuric acid (0.1 to 0.5 cc of the commonly used to 10 per cent., added to 100 cc distilled water) it makes the *Koch-bacillus* appear to have a more distinctly blue colour. Nevertheless, the results are uneven and different.

If, on the other hand, sulphuric acid is neutralized so that to 90 cc of distilled water are added 1 cc N 1 H_2SO_4 and 1 cc N 1 KOH, and then 10 cc *Nachtblau* alcohol, the bacilli are nicely stained. If a little more alkali than acid is added, the bacteria are more darkly stained, if a little more acid than alkali, lighter. In

the former case the dye-liquid keeps for a shorter time, in the latter for a longer.

Nachtblau does not dissolve in 0.9 per cent. (physiological) NaCl but in such of 0.1 per cent. The latter solution stains Koch's bacillus faintly blue. Nachtblau behaves in the same way in weak solutions of some other chlorides and nitrates, whereas in diluted solutions of some sulphates it brings about a considerably stronger staining of the tubercle bacilli.

The dye-liquid can thus be obtained of a diluted potassium sulphate solution (about 0.04 per cent.) to which is added Nachtblau alcohol. This solution is comparatively more stable. According as the saline contents increases, the solution grows less stable. On heating over a flame to the boiling point, the result is, however, not so fine and beautiful as with fainter and longer heating in a stain bath. Decolorization with hydrochloric alcohol also occasions some difficulties. The preparation is certainly discoloured, but when this is afterwards washed with distilled water it grows again faintly blue. Decolorization with 8 per cent. nitric acid and 70 per cent. alcohol is better, especially if the preparation prior to the acid treatment has been differentiated in distilled water.

This sulphate-Nachtblau fluid has here been experimentally used for staining several preparations in a colour-bath with the aid of a Klein's stain-basket according to a process described in detail on p. 16. The solution in question can stand repeated heating without being destroyed.

Carbolic acid may be exchanged for some fatty acid alkali salts, e. g. potassium acetate, sodium propionate, and sodium butyrate. It has not yet been finally tested which of these gives the best results. The sodium acetate, which is most easily available, has here been mostly used. These salts are soluble in 95 per cent. alcohol and it has therefore been possible to let them form an ingredient of the saturated Nachtblau stock solutions used for the *simultaneous method* (vide infra). These stock solutions, from which the staining solutions are prepared through adding 10 cc to 90 cc distilled water, contain also a small quantity of Bismarckbraun, and appear to be stable for several months. But it is probable that Bismarckbraun in the alcohol stock solution is gradually weakened, and that in such case it will be more advantageous in some form to dissolve a corresponding quantity of Bismarckbraun in

the ready staining fluid. The observation period has not been sufficiently long. With this staining fluid there is thus no need of any particular fluid for contrast staining, so that one may, without washing the preparation in distilled water, slake and dry after decolorization with nitric acid and 70 per cent. alcohol. The preparations have nevertheless been found to keep for quite a long time without the blue bacteria against the yellow background being enfeebled. This staining fluid is not usable for basket-staining, since with repeated heating it seems to undergo some changes.

Anilinum liquidum is likewise usable as a dispersion agent when it is a question of Nachtblau. Ehrlich's aniline stain is said to remain stable for about 10 days. As regards Nachtblau, it will not be necessary, as Ehrlich did, to start with a concentrated and filtered 3 to 4 per cent. aniline solution. It will be sufficient with 1 cc Anilinum liquidum dissolved in 90 cc distilled water + 10 cc Nachtblau stock solution, or 1 cc Anilinum liquidum dissolved in 10 cc Nachtblau alcohol + 90 cc distilled water, which is quicker, since the aniline is better dissolved in alcohol than in distilled water. To this can subsequently, for simultaneous staining fluid, be pipetted 2 cc 2 per cent. glycerin solution of Bismarckbraun. This staining fluid has proved stable for a month or so. The less aniline used, the better the stability. A glycerin solution of Bismarckbraun has been used, since this has proved to be stable and can therefore be used as a stock solution.

CHAPTER IV.

Successive Method.

Stock-, staining-, and decolorising solutions, and counter-stains.

I a. *Saturated stock solution.*

- | | |
|---|--------|
| 1) Nachtblau (Grübler & Co., Leipzig) | 5 g |
| 2) 95 per cent. alcohol | 100 cc |

— The flask is shaken repeatedly during the day and left, at least overnight, for sedimentation of any possibly undissolved matter.

I b. *Staining solution.*

1) Distilled water.....	90 cc
2) 10 per cent. potassium hydrate ¹	max. 0.2 cc
3) Phenolum liquefactum.....	2.5 cc
4) Of the saturated stock solution (I a.)	10 cc
— The saturated Nachtblau solution is taken by pipette from the upper layer, not from the bottom. It is added after the potassium hydrate and the phenol have been dissolved in the water.	

II a. *Saturated stock solution.*

1) Nachtblau.....	5 g
2) Phenolum liquefactum.....	25 cc
3) 95 per cent. alcohol.....	75 cc
— The Nachtblau is added only when the phenol has been dissolved. Then the flask is shaken etc.	

II b. *Staining solution.*

1) Distilled water.....	90 cc
2) Of the saturated stock solution (II b.).....	10 cc

III. *Decolorising solution.*

1) 25 % hydrochloric acid	3 cc
2) 70 (sic!) per cent. alcohol.....	100 cc
— Hydrochloric 70 per cent. alcohol discolours more strongly than hydrochloric 95 per cent. alcohol. In discolouring with the latter some other bacterial forms also retain their blue colour.	

IV. *Counter stains.*A. *Pyronin.*

1) Distilled water.....	100 cc
2) Pyronin	0.25 cc
3) Phenolum crystallisatum.....	0.5 g
— The pyronin is first completely dissolved in the water after which the phenol is added.	

B. *Neutral-red.*

1) Distilled water.....	100 cc
2) 1 per cent. acetic acid.....	0.2 cc
3) Neutral red.....	0.1 g

¹ Not absolutely necessary. If potassium hydrate is added the bacilli are stained more intensely when the sort of Nachtblau is used which easily dissolves in distilled water.

C. *Carbol-fuchsin*.

1) Distilled water.....	95 cc
2) Concentrated carbol-fuchsin.....	5 cc

D. *Vesuvium or Bismarckbraun*.¹

1) Distilled water.....	100 cc
2) Of the two dye-stuffs respectively.....	2 g

Staining procedure.

Films.

Thin films, well air-dried and fixed in the flame, are treated as follows:

- 1) Pour plenty of *Nachtblau* staining solution (I b. or II b.) on the slide. Heat carefully over the flame until boiling. Allow the stain to act for about 5 minutes during which period the slide will have time to cool.
- 2) Pour off stain.
- 3) Decolorise with solution III until no more blue clouds are given off.
- 4) Wash in distilled water.
- 5) Counter-stain with any of the five above solutions: for 5—10 seconds if neutral-red, carbol-fuchsin, vesuvin, and Bismarckbraun are used; pyronin requires a somewhat longer time (15 seconds or even more).
- 6) Wash in water, blot and dry.

Sections.

- 1) Place the slide on which the section has been well fixed for about 5 minutes in the usual manner in a cuvette containing xylol in order to remove the paraffin.
- 2) Wash consecutively in 95, 80, and 65 per cent. alcohol.
- 3) Wash in distilled water.
- 4) Blot, slightly, with filter-paper.
- 5) Pour on *Nachtblau* staining solution (I b. or II b.).
- 6) Heat three times until vaporization sets in, and allow the stain to act for about 5 minutes after the last heating.
- 7) When the slide has cooled, decolorise the section with solution III and wash in distilled water. Counter-stain with pyronin for 2 minutes.
- 8) Wash in distilled water, blot with filter-paper, and dry completely over the flame.
- 9) Mount in cedar oil.

¹ Even crysoidin had been used at the time of my publication in 1941 but the tubercle bacilli assumed a green colour. With the use of the procedure published, in 1944, by Quandt (31) they become more blue-stained. Cf. p. 24.

Nachtblau staining (successive method) of several films at one and the same time.

I. *Stock solutions.*

- A. Saturated solution of Nachtblau in 95 per cent. alcohol.
- B. 4 per cent. potassium sulphate solution (the salt dissolves best in hot distilled water).

II. *Staining solution.*

- 1) Distilled water..... 270 cc
- 2) Stock solution I B..... 3 cc
- 3) Stock solution I A..... 30 cc

III. *Decolorising solutions.*

- A. 8 per cent. nitric acid (approximately) followed by
- B. 70 per cent. alcohol.

IV. *Counter-stain: Pyronin.*

Staining procedure.

(Text-plate.)

1) Place the air-dried and well fixed films in a Klein's stain-basket (Fig. 1) and immerse the basket in a cup containing the staining solution (Fig. 2).

2) Put the cup with the basket down in a water-bath (Fig. 3)¹ and see that the water reaches above the stain in the cup.

3) Heat the water-bath until the temperature of the staining solution reaches 75°—80° C.

4) Raise the cup and allow the staining solution to cool to 40°—50° C. (Fig. 4).

5) Lift out the basket and wash off the stain in two cups with water (Figs. 5 and 6) for a few seconds, and 1 to 2 minutes respectively.

6) Then expose the films to 8 per cent. (approximately) nitric acid for 15 seconds (Fig. 7).

7) After that, for decolorization, let the basket remain in two cups with 70 per cent. alcohol (Figs. 8 and 9) for 3 and 2 minutes (or more) respectively.

8) When the films appear to be completely decolorised, wash quickly in distilled water (Fig. 10).

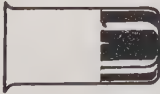
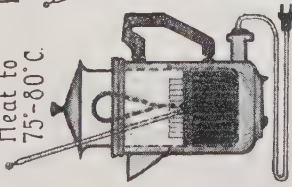
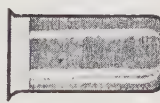
9) Counter-stain with pyronin for 15 seconds or even more (Fig. 11).

10) Wash in distilled water (Fig. 12), remove the slides, blot and dry.

When the Nachtblau solution has to be used for staining again, as much distilled water must be added to the quantity as has been lost through vaporization.

¹ We used as water-bath an electrical boiler of aluminium in which the lid had been cut out so that the stain-cup can scarcely get through. Thus, the latter remains firm. Klein's stain-basket is obtainable at KIFA, Stockholm.

Nachtblau staining (successive method)
of several films at one and the same time.

1.	Put slides in	2.	Put basket in	3.	Heat to 75°-80° C.	4.	Raise cup; cool to 40°-50° C.	5.	Wash briefly	6.	Wash 1-2 min.	7.	15 sec.	8.	3 min.	9.	2 min.	10.	Wash	11.	Counter-stain 15-30 sec.	12.	Wash
																							
	Klein's stain-solution basket		Nacht-blau solution		Electric boiler (even common waterbath useful)				Dist. water I		Dist. water II		8% nitric acid		70% alcohol I		70% alcohol II		Dist. water		Pyronin		Dist. water

CHAPTER V.

Simultaneous Method.**Stock-, staining-, and decolorising solutions.****I. Saturated stock solution.**

- | | |
|---|--------|
| 1) Potassium acetate, or sodium propionate, or sodium butyrate. | 1 g |
| 2) 95 per cent. alcohol..... | 100 cc |
| 3) Nachtblau..... | 5 g |
| 4) Bismarckbraun..... | 0.4 g |

— The above salts are first dissolved in 100 cc alcohol.
After that Nachtblau and Bismarckbraun are added.
The flask is shaken repeatedly etc.

II. Staining solution.

- | | |
|-------------------------------|-------|
| 1) Distilled water..... | 90 cc |
| 2) Of the stock solution..... | 10 cc |

III. Decolorising solutions.

- | | |
|--|--------|
| A. 1) 25 per cent. (approximately) nitric acid | 4 cc |
| 2) 70 per cent. alcohol | 100 cc |
| or | |

B. 8 per cent. (approximately) nitric acid followed by

C. 70 per cent. alcohol.

Staining procedure.**Films.**

Thin films, well air-dried and fixed in the flame, are treated as follows:

1) Pour plenty of Nachtblau staining solution (II) on the slide. Heat carefully over the flame until boiling. Allow the stain to act for about 5 minutes during which period the slide will have time to cool.

2) Pour off stain.

3) Wash in distilled water.

4 a) Decolorise with nitric acid alcohol (III A.) until no more blue clouds are given off.

5) Blot well with filter-paper (without previous washing in water) and dry over the flame.

or

4 b) Dip the slide for 15 seconds in solution III B.

5) Decolorise directly (without previous washing in water) with solution III C. until no more blue clouds are given off.

6) Blot well with filter-paper (without previous washing in water) and dry over the flame.

Sections.

1) Place the slide on which the section has been well fixed for about 5 minutes in the usual manner in a cuvette containing xylol in order to remove the paraffin.

2) Wash consecutively in 95, 80, and 65 per cent. alcohol.

3) Wash in distilled water.

4) Blot slightly with filter-paper.

5) Pour on plenty of Nachtblau staining solution (II).

6) Heat three times until vaporization sets in and allow the stain to act for about 5 minutes after heating.

7) When the slide has cooled pour off the stain and wash in distilled water.

8 a) Decolorise with nitric acid alcohol (III A.) until no more blue clouds are given off.

9) Blot well with filter-paper (without previous washing in water) and dry over the flame.

10) Mount in cedar oil.

or

8 b) Dip the slide for 15 seconds in solutions III B.

9) Decolorise directly (without previous washing in water) with solution III C. until no more blue clouds are given off.

10) Blot well with filter-paper (without previous washing in water) and dry over the flame.

11) Mount in cedar oil.

NOTE.

Previously we employed the simultaneous method by using the following solutions:

I. *Stock solutions.*

A. Saturated alcoholic Nachtblau solution.

B. Anilinum liquidum.

C. 2 per cent. Bismarckbraun-glycerin solution.

II. *Staining solution.*

1) Nachtblau stock solution (I A.)..... 10 cc

2) Anilinum liquidum (I B.)..... 1 cc

3) Distilled water..... 90 cc

4) 2 per cent. Bismarckbraun-glycerin solution (I C.)..... 2 cc

Decolorising solutions and staining procedure as above. The said staining solution retains, however, its efficacy only for about one month.

CHAPTER VI.

Experiences with regard to the Tubercle Micro-organism obtained by the New Staining Method.

As reported above (Chapter II), I tried the staining first on smears from a Koch-bacillus culture and of sputa originating from patients suffering from pulmonary tuberculosis, after which I continued with tests on different tuberculous material, in films and sections.

Since 1939 I have very likely examined several thousands of slides. My experience, from a *practical* point of view definite, is: That the tubercle bacilli appear more numerous in preparations stained with Nachtblau than in those stained according to the Ziehl-Neelsen method (where the counter-stain conceals several of the specimens), seem to be larger, more strongly stained (dark-blue), and more distinctly standing out against the background (red or yellow-brown), resulting in the fact that they are more easily detected, so that a great deal of time in laboratory routine work is saved.

Both in Sweden and abroad many physicians have tried the method in question (successive¹ procedure, by the use of carbol-Nachtblau).

The majority of the results of the Swedish tests, carried out in laboratories, hospitals, sanatoria, and in private practice, have been reported to me from personal information. But there are some research-workers in this country and abroad who have put their experiences in print.

All of them seem to agree with regard to what is said above about tubercle bacilli stained by the new method (Kristenson, Larsen, Hamperl, Rubarth and Agrell, Bertolini, Tegström, Roulet, Dissmann, Anna Andersson, Hollström, Quandt, Nyman, Linell and Vahlne, Wihman, Grönwall and Zetterberg).

Kristenson (26), counting the bacilli in the same fields of vision in films of tuberculous sputa, previously stained by Ziehl-Neelsen and afterwards with Nachtblau, found the number of the germs to be equal. He says that the appearance of the bacilli of being less numerous in the Ziehl-Neelsen preparations is probably due to the fact

¹ The more recently elaborated simultaneous procedure has only been used by myself and a few others.

that, especially in thick films, a good many of the germs are covered by the methylene-blue, which allows them to escape the eye, and moreover, to an inferior combination of colours. Kristenson finds the Nachtblau method superior to the Ziehl-Neelsen.

Larsen (27) states that the Nachtblau method is superior to the Ziehl-Neelsen but inferior to that by the use of fluorescence-microscope.¹

Hamperl (14) emphasizes especially the bacilli's appearing thicker when the new method is used.

Rubarth and Agrell (39) state regarding the Nachtblau method used by them in the examination of material infected by human, bovine, and avian tubercle bacilli, that it has facilitated the pathohistological tuberculosis diagnostic, especially in cases of tuberculosis in horse and dog, where as a rule the germs occur sparsely, and that the results obtained are more to be trusted. For the said reasons the authors mostly use only the new method in their diagnostical work (at the State Veterinary Institute, in Stockholm), in order to prove directly the presence of tubercle bacilli in tissue.

In this connection it should be mentioned that, since several years, the Nachtblau method has been used instead of the Ziehl-Neelsen in the routine-work at the Institute of Pathology, at Upsala (personal communication from its Director, Professor R. Fåhræus).

Bertolini (2) considers that the Nachtblau method is superior to Ziehl-Neelsen's.

Tegström (42), who examined smears prepared from scrapings from a tuberculous eye-ulcer (accidental laboratory infection) says: "In a Ziehl-Neelsen-stained film could be detected a few acid-fast rods not very typical of tubercle bacilli. The same film was then stained according to Hallberg's Nachtblau method. There was then found quite a large number of acid-fast rods, which morphologically and tinctorially entirely corresponded to the bacilli of Koch. — In one field of vision 15—20 specimens could be seen. — Also in another film which had been stained directly by the Hallberg method, numerous similar bacilli were found."

Roulet (38) was able to prove the correctness, in all details of the

¹ It may be recalled that the fluorescence-microscopical apparatus is an expensive installation that not everybody can afford. Moreover, one feels some uncertainty whether all the rods seen under the fluorescence-microscope may really be considered as tubercle bacilli.

writer's statements, in 1941, except with regard to the stability of the Nachtblau staining solutions (vide the explanation of the difference of opinion in Chapt. III). He recalls some important experiences with regard to the combination of colours in Ziehl-Neelsen's method: The examination of sections becomes very tiring after a long search, on account of the facts that the ground tone of the tissue is seldom really free from rose-red shades, and that the not very sharp contrasts between the red luminous bacilli, the pale-violet nuclei, and the changing of the background from rose to slightly violet call for greater attention. Also the counter-staining of a section with methylene-blue — as generally used in Ziehl-Neelsen's method — possesses pronounced disadvantages, which are specially due to the violet-red shades of the section's background.

Roulet, from different points of view, concludes that the Nachtblau method is superior to that of Ziehl-Neelsen and is convinced "that it will, for daily use, gain entrance to an Institute of Pathology which has no fluorescence-microscopical apparatus".

Dissmann (4), among all foreign research workers, seems to have carried out the most far-reaching tests with the Nachtblau method.

Apart from the above staining properties of the tubercle bacilli Dissmann was able to state that also their granulated forms, often difficult to detect when Ziehl-Neelsen is used, appeared "always extraordinarily pretty". He was, furthermore, already at the first comparison between the two methods in question, struck by the Nachtblau staining giving a much greater surplus of bacilli in the tuberculous sputa examined.

On closer examination — first counting the Ziehl-Neelsen-stained bacilli in a greater number of fields of vision, and then counting them in the same fields, since the films had been stained afterwards with Nachtblau — Dissmann found that the number of tubercle bacilli in the thicker preparations was 3 to 6 times greater in favour of the Nachtblau method while, on the other hand, this superiority amounted to but a few per cent, in the thinnest ones. The use of Bismarckbraun as counter-stain instead of methylene-blue resulted in a certain, though not great, superiority of the Nachtblau method in thick films, and when counter-stain was omitted the difference in number became still less pronounced. Thus, the counter-stain employed is of great importance. Several authors, Dissmann says, consider the methylene-blue inferior to other counter-stains because

the red rods are covered and wiped out through both colours' belonging to similar spectral areas.

Furthermore, Dissmann compared the influence of direct daylight and sunlight on tubercle bacilli in films of sputa of different thickness stained by Ziehl-Neelsen and Nachtblau respectively. He was then able to state that, e.g. in the films exposed to day-light for 3 days, 43 per cent. of the previously counted bacilli had disappeared (bleached away) in the Ziehl-Neelsen slides but only 7 per cent. in those stained with Nachtblau; with regard to the films exposed to sunlight, he was able after 8 days to state that in those of the former group nearly 50 times more bacilli had disappeared than in those of the latter.

Dissmann considers Nachtblau to be a superior dye to fuchsin. The contrast of the blue-stained tubercle bacilli against a red or yellow background, instead of red-stained ones against a blue, even yellow one, is better. This fact, together with the bacilli's appearing larger and more easy to detect also in thicker films (containing a greater number of bacteria) results in the use of the Nachtblau staining saving much time in the routine work. While Koch reckoned with at least 20 minutes for searching through a Ziehl-Neelsen-stained sputum-film, Dissmann is of the opinion that for preparations, stained according to the Nachtblau method, 3-4 minutes might be enough and that, without any risk, the examination can be entrusted to less experienced helpers.

Finally, Dissmann emphasizes that the Nachtblau method in a qualitative and quantitative regard is superior to Ziehl-Neelsen's, and expresses the hope that it will be the method of the future.

Anna Andersson (1), who like Kristenson, Roulet, Dissmann, and others asserts the draw-back of using methylene-blue as counter-stain in the Ziehl-Neelsen method, examined a large number of slides of tuberculous sputa, by use of the fluorescence-microscope and according to the Ziehl-Neelsen and the Nachtblau methods, and was able to state that the results obtained by the staining with Nachtblau were superior to those obtained by the Ziehl-Neelsen, but inferior to those which the use of the fluorescence-microscope gave.

Hollström (19) emphasizes that his findings of tubercle bacilli and acid-fast fungus cells of yeast-oidium-type were enabled, or at least facilitated, by the use of the new method, where the counter-stain

does not spoil their appearance.¹ With regard to the fungus cells' taking the Nachtblau stain, vide further p. 30.

Quandt (31) who tried the new method on tuberculous sputa and sections used as counter-stain crysoidin, which in sections was rapidly washed away in water, but not at all in films, as the writer did (Cf. the foot-note on p. 15). By counting a large number of corresponding fields of vision in preparations stained by Ziehl-Neelsen and according to the Nachtblau method, Quandt was able to state a surplus of 27 per cent. tubercle bacilli in favour of the new method, both regarding films and sections (from cavernous pulmonary tissue).

Nyman, Linell, and Vahlne (30) state that the fluorescence-microscopical method is somewhat superior to the Nachtblau staining but that, on the other hand, the latter gives better results than Ziehl-Neelsen's, and is a simple and handy method, apt to be used by practitioners and at smaller hospitals and institutions where it is found impossible to procure such a comparatively complicated apparatus as the fluorescence-microscope is.

Wihman (47), who for examination of tuberculous sputa compared the results obtained by a new concentration procedure with those in common direct films and in his work used both Ziehl-Neelsen and Nachtblau stain, found the latter method somewhat superior.

Grönwall and Zetterberg (10) are of the opinion that the superiority of the Nachtblau method to Ziehl-Neelsen's is due to better conditions of contrast, which make it easier to see the tubercle bacilli.

Several findings of a more *scientific* nature have been obtained by the use of the Nachtblau method on material where the Ziehl-Neelsen had given negative results.

Thus, Hellerström and Hallberg (17) found in a case, clinically and anatomo-pathologically greatly suspected of being lupus vulgaris, a few acid-fast tubercle bacilli-like rods — in another analogous case negative results were obtained, both after staining by Ziehl-Neelsen and with Nachtblau.

Schaumann (40), and Schaumann and Hallberg (41), were able to prove in organs from cases of Lymphogranulomatosis benigna (Schaumann's disease) the occurrence of fungus cells, which they

¹ When stained according to the Ziehl-Neelsen, without counter-staining, the fungus shows as a rule in fresh cultures a faint rose-colour, which when methylene-blue follows is concealed for the greater part.

assert as being quite similar to those described by Reenstierna in 1912, and, furthermore, in the organs in question, apart from such fungus cells, also acid-fast rods with the same morphological and tinctorial properties as those of Koch's bacillus. These findings might be considered as especially interesting in view of the fact that L. B. is by Schaumann regarded as being a disease of a tuberculous nature, though it does not seem to cause positive tuberculin reaction.

Hollström (20) reports on a case of L. B. where in a lymph gland, extirpated during an operation from the neighbourhood of the gall-bladder, numerous fungus cells of the above type and solitary, slightly acid-fast rods could be proved. In the discussion on the etiology of L. B. the author emphasizes the possibility of the origin of the rods from the fungus cells and of the tuberculous nature of the disease.

Fungus cells of the same appearance, together with tubercle bacilli, were, during the routine-work at the Institute of Hygiene and Bacteriology, at Uppsala, occasionally detected in glandular pus from guinea-pigs, which six weeks earlier had been inoculated with tuberculous material (sputa, uretero-catheterized urines, and cerebro-spinal fluid), and, furthermore, very small acid-fast granules were often found in the pus (perhaps transition forms to Fontes' filterable tuberculosis virus).

CHAPTER VII.

Application of the Nachtblau Method to Staining other Acid-fast Micro-organisms, that of Leprosy, etc.

The causative agent of Hansen's disease.

To my aforesaid paper of 1941 on the new method for staining tubercle bacilli Reenstierna had added a short note, together with a plate, on its use for staining leprosy bacilli (in nasal smears, films of blood, and sections of lepromata). It was then stated, as was to be expected, that the latter, in the same way as the former, take a dark-blue colour, appear larger and more distinct¹, and for that reason, when occurring sparsely in the material,

¹ These facts did not at all become manifest from the microphotographs of the plate mentioned, which had been taken by the use of the German

are easier to discover than in corresponding films stained according to Ziehl-Neelsen.

Moreover, it was mentioned in the said note that by the use of the Nachtblau method, it had been possible, in 1939, to detect in thick films of blood taken from the arm vein of a Swedish leper (nodular form in a moderately acute stage), besides sporadic Hansen's bacilli, acid-fast, oval, and pear-shaped bodies (Pl. IV, fig. 1), resembling the cellules of which was composed a fungus isolated, in 1912, by Reenstierna (32) from the circulating blood of another Swedish leper (nodular type in a very acute stage), an organism which according to Reenstierna is the maternal fungus of Hansen's bacillus. Thus e. g. he considers the spindle-shaped conglomerations of leprosy bacilli as nothing else but the differentiated contents of oval fungus cells (36) which, before bursting, generally swell. From the blood of the patient of 1939 a mycotic micro-organism was isolated, which was composed of ovoid elements like those found in the direct blood films. Only parts of it showed, both by Ziehl-Neelsen and Nachtblau staining, a certain degree of acid-fastness. It was further noticed i. a. that similar fungus-cells occurred in leprous nasal smears, originating from lepromatous patients in different countries, and that the same were partly acid-fast (Pl. IV, fig. 3).

Finally, there was reported on the behaviour of the Nachtblau staining to a queer micro-organism which Reenstierna, in 1924, isolated from the cerebro-spinal fluid of a third Swedish leper (nodular form in a fairly acute stage). Thus, in films from the culture, stained with Ziehl-Neelsen in the said year and stained afterwards with Nachtblau, in 1941, there appeared, when hydrochloric 96 per cent. alcohol was used as a decolorising solution, instead of the usual 70 per cent., a number of dark-blue corpuscles in between the counter-stained specimens of micro-organism (Pl. IV, fig. 4).

Roulet (38) who tried the Nachtblau stain on different acid-fast bacilli says with regard to its use on Hansen's bacillus: "Also the detecting of leprosy bacilli succeeded — in agreement with the statement of J. Reenstierna — very easily, even in preparations which for years had been kept in preserving solutions."

Since the appearance of the aforesaid note by Reenstierna I

"Agfacolor Neu" film (Cf. the foot-note of Explanation of Pl. I). But in a more recent paper by Reenstierna (36) we find a picture (section of leproma) where full justice is done to the method.

have made a closer study of the value of the Nachtblau method for staining the leprosy micro-organism (in films from cultures, nasal smears, thick blood films, and sections from lepromata). And then I have not only used the successive procedure, as described in 1941, but also the simultaneous one.

Thus, two cultures, kept at the Upsala Institute, were examined: The one of a micro-organism which Kedrowski (22), in 1900, isolated from a leproma of a Russian patient, the other of Reenstierna's aforesaid micro-organism of 1912, both at present growing on the surface of glycerin bouillon where they form greyish and greyish-yellow pellicles, respectively, composed of rods and filaments, which behave partly as acid-fast, partly as non-acid-fast when stained by Ziehl-Neelsen. Thus it was several years ago and thus it is now. On examining such films as were stained afterwards with Nachtblau one gets a certain conception that acid-fast bacterial elements appear more numerous.

Regarding leprosy bacilli in thin nasal smears, it may be debated whether the Nachtblau method is superior to the Ziehl-Neelsen, apart from their looking larger and more distinctly stained. In thicker films, on the other hand, they appear definitely in greater number when the former method is used, due to the fact that the methylene-blue of the latter covers a good many of the red-stained bacilli and also because the red touch which this dye-stuff, at least sometimes, shows, exercises a disturbing influence on the tracing of the micro-organism in question. Cf. the statements by Kristenson, Roulet, Dissmann, and others as to staining tubercle bacilli with Ziehl-Neelsen (Chapt. VI).

In 1942 the writer examined some thick films made from the circulating blood of a fourth Swedish leper (nodular form in a not very active stage). In one of them it was possible to detect, by the use of the Nachtblau staining (successive procedure), an spindle-shaped acid-fast fungus-cell (Pl. IV, fig. 2) closely resembling those previously found in the blood by Reenstierna, Walker (46), and probably also by Bosma (3). Its culture was not successful.

With regard to sections of lepromata, it is a well-known fact that their staining with Ziehl-Neelsen gives very capricious results. Either the preparations are underdecolorised, so that also the tissue in a disturbing manner appears red, or they are overdecolorised with the result that a large number of the leprosy bacilli — which more

easily lose the fuchsin taken, contrary to tubercle bacilli — remain faintly or not at all stained.

Illuminating for the latter case is the following, carried out in 1945: A very skilful technician, experienced in the matter concerned, stained and re-stained according to Ziehl-Neelsen, a good many sections from lepromata originating from different Swedish patients. The first group consisted of sections freshly cut from nodules which for fully twenty years had remained embodied in paraffin, while the second consisted of sections which, stained with Ziehl-Neelsen twenty years ago, had since remained mounted in Canada balsam.

In some of the sections belonging to the first group the bacilli were distinctly stained; in all of the second group, on the other hand, the bacillus-staining failed more or less completely. In the unsuccessful preparations of both groups it was, however, possible to detect balls of bacilli, some faintly rose-coloured, others quite unstained and refractive.

The sections were afterwards stained according to the Nachtblau method — successive as well as simultaneous procedure — with the following results: All the preparations of the first group showed clearly bacilli, stained very distinctly dark-blue, as previously unstained ones do (Pl. III, figs. 1 and 2); in those of the second group the bacilli had also throughout nicely taken the Nachtblau stain, with the modification, however, that in some odd spots of a few sections the colour of the micro-organisms appeared somewhat faded and the outlines of the bacterial bodies were less sharp.

Thus, with regard to staining sections of lepromata, the Nachtblau method must be considered as superior to that of Ziehl-Neelsen.

Moiser (28), for many years Medical Superintendent of the Ngomahuru Leper Hospital at Fort Victoria, Southern Rhodesia, was able to state the presence of "Oval Bodies" up to 70 per cent. in the guts and faeces of cockroaches (mostly of the species *Blatella germanica*), whether caught inside the hospital grounds or taken from Kraals in the native reserve. These "Oval Bodies", which Moiser emphasizes to be identical with cells of Reenstierna's aforesaid "Maternal Fungus", isolated, in 1912, from the blood of a Swedish leper, are greatly varying in size, and the great majority of them acid-fast when stained according to Ziehl-Neelsen. Smears (from female cockroaches), stained in that way, which had been sent to Upsala, were

stained afterwards with *Nachtblau* and in doing so proved to be acidfast even according to this method.

The causative agents of Johne's, Traum's, and Gellerstedt's diseases, a few other pathogenic organisms, as well as some so-called saprophytes.

Johne's disease (21), attacking sheep, horses, and cattle, displays a chronic hypertrophic enteritis with mesenteric glandular swellings, which alterations contain enormous masses of acid-fast rods (Ziehl-Neelsen's method). Gellerstedt, examining intestinal sections, proved that the bacilli also showed acid-fastness when stained with *Nachtblau* (Pl. IV, fig. 5).

Traum (43) described a disease, in the American literature passing under the name of "skin lesions in tuberculin reacting cattle", which manifests itself by cutaneous and subcutaneous resistances of a more or less tuberculoid structure, and containing acid-fast rods (Ziehl-Neelsen). Hedström, Gellerstedt, and myself were able to prove that the bacilli, when *Nachtblau* stain was used, even behaved acid-fast (Pl. IV, fig. 7). Moreover, Hedström (16) succeeded in isolating from the skin lesions and corresponding lymph glands, besides bacilli, a yeast-like fungus which showed acid-fastness, stronger when stained with *Nachtblau*. Regarding its stainableness vide further p. 31. — The said fungus inoculated into cattle gave rise to pronounced changes of tissue like those in Traum's disease and, furthermore, with rather great certainty tuberculine allergy, for which reason the author considers it to be of etiological importance in the disease concerned. — Finally, it should be stated that, in a section of a fresh skin nodule which Hedström had placed at my disposal and which had been stained with *Nachtblau*, I succeeded — after a long search — to discover, besides bacilli, some fungus cells, also behaving acid-fast (Pl. IV, fig. 8).

Gellerstedt (7, 8, 9) who described a tuberculoid lesion of the skin (7 cases) in man, anatomo-pathologically much resembling the above skin disease in cattle and caused by a somewhat pleomorphic micro-organism, chiefly acid-fast rods (Pl. IV, fig. 6) — no doubt a primary exogenous infection —, used on staining the bacilli besides Ziehl-Neelsen's method also that with *Nachtblau*, about which latter he

states its being greatly superior to the Ziehl-Neelsen method, as regards distinctness.

Rubart and Agrell (39) stated that the Nachtblau method gave prettily positive results with regard to organs infected by *B. paratuberculosis bovis* as well as in a case of infection (in cat) by *Streptotrix canis*.

Roulet (38) tried with positive results the Nachtblau method on frog- and turtle bacilli, as well as on smegma-, water-, "Timothy-Grass"-, and other bacteria of saprophytic nature. Nyman, Linell, and Vahlne report (30) on a saprophytic bacillus which, not only according to Ziehl-Neelsen, but even with Nachtblau, showed acid-fastness. Also at the Institute of Hygiene and Bacteriology in Upsala several strains of saprophytes (in all about 20) were stained with Nachtblau and then proved to be acid-fast.

ADDENDUM.

Fungi.¹

Oidium (*Monilia*, *Candida*). To what has been said above regarding this fungus, should be added the following. In cultures of the same one meets with two principal forms, the yeast-like blastospores and the longer pseudomycelia. Stained with Nachtblau the blastospores behave chiefly as acid-fast, while those cells which are in a stage of transformation into pseudomycelium, show a clearly transitory colour (violet e. g. when pyronin had been used as counter-stain). The latter cells lose, however, their acid-fastness in the same degree as they are transformed into typical pseudomycelium. This takes only the counter-stain. The same principle holds good even for older cultures; only there appears here a third type of fungus elements, clamydospores. These are larger, round formations, which as a rule behave non-acid-fast. Only in very solitary cases one finds a clamydospore showing a slightly transitory colour or an acido-resistance to a certain degree (with a touch of light-blue). — In this connection should be recalled Hollström's observations in *Monilia* cultures (19) to the effect that the acid-fastness, strongly occurring in younger fungus elements, is more or less lost when the

¹ This reconnoitring investigation was carried out in collaboration with the Esthonian mycologist Dr. H. Paldrok.

cultures grew older (Pl. II, figs. 9 and 10), observations agreeing with those made by Hedström (16) and by Törnell (44, 45), with regard to similar fungi isolated by them from skin nodules in Traum's disease and from sputa of patients suffering from "thresher's lung", respectively.

Actinomyces. The principal forms which the mycelium, when breaking down, delivers are segments, rods, and coccical forms. The latter two we found acid-fast, stained with Nachtblau, while the one-cell mycelium and the segments always behaved as non-acid-fast. — According to the biographical statements the acid-fastness of the different species of Actinomycetes varies considerably.

Common moulds.

It may be generally stated that as a rule neither the mycelial branches nor the conidia-bearing system seems to be acid-fast. In some of these, however, larger or smaller structureless corpuscles were noticed which, in conformity with some solitary branches, showed complete acid-fastness.

Penicillium. Apart from the fungus elements mentioned, the newly formed conidia did not take the Nachtblau stain. A transitory colour (violet when pyronin had been used as counter-stain) appeared in the conidial chains only at some distance from the sterigmata of e. g. three or four cells. This colour, comprising several cells, increased in intensity and, finally, changed into dark-blue in the ripe conidia.

Aspergillus. The borders of colour change here appeared to be somewhat less sharp. Furthermore, the transitory colour in some of the conidial chains of the same head began already in the cells, close to the sterigmata, in other chains only in more distant¹ cells. As in the case of *Penicillium*, the sterigmata and the newly formed conidia remained unstained. The ascospores appeared as (light-)blue, structureless (hyaline) globules, enveloped by an also structureless capsule, which was unstained or only slightly counter-stained. Embodied in blood serum, this capsule was liable to disappear completely from view.

¹ This difference in equal fungus elements (*Penicillium* and *Aspergillus*) we are unable to explain in regard to the taking of the Nachtblau stain.

On germination the conidia of *Penicillium* and *Aspergillus* lost their acid-fastness, and that at the same rate as the germination progressed. The more swollen the cells grew, the lighter they were, and when they reached the stage of tubular formation, they merely took the counter-stain.

Mucor. The sporangiospores showed a slightly transitory stain (violet with a touch of brown when pyronin was used as counter-stain), while the sporangia, copulation branches, and progametangia etc. did so until their stage of young zygotes only took the counter-stain. We have so far not succeeded in staining a fully developed zygote.

By the use of the *Nachtblau* method on different spore-bearing bacteria e. g. *B. anthracis*, *B. subtilis*, and *B. perfringens*, the spores, as it seems, in a certain stage of their developement take a dark-blue stain.

Finally, it should be stated that several investigations have been started in order to clear up various obscure problems with regard to the real morphology of the causative agents of tuberculosis, leprosy, and other diseases: detecting other forms than the classical bacilli which all, no doubt, are merely evolutionary stages, originating from lower, commonly occurring fungi (the aforesaid of yeast-oidium-type and possibly others).

Furthermore, the writer wishes to emphasize that the *Nachtblau* method as described here before is not to be considered as a definitely closed chapter, as, no doubt, modifications of the same will prove even more suitable.

The expenses for the research-work have been defrayed by the Faculty of Medicine in Upsala and the Swedish Anti-Tuberculosis League. I wish to convey my heartiest thanks to these bodies.

Summary.

The writer has been occupied since 1939 in elaborating a new method for staining tubercle bacilli by using the dye *Nachtblau* (Grübler & Co.), which in water solution behaves as a colloid. Of the said matter there issued from the factories numbers which

easily dissolve in distilled water, and others which are less soluble. Carbolic staining solutions prepared from the former sort keep their efficacy for a long time, while solutions prepared from the latter remain stable only for a short period.

The staining according to the Nachtblau method can be carried out by two different procedures: the *successive* and the *simultaneous*. In the former the Nachtblau is followed by counter-stain; in the latter the staining solution contains both Nachtblau and counter-stain. Staining several films at one and the same time as well as staining afterwards with positive results of preparations previously stained by Ziehl-Neelsen can easily be done.

The writer has tried the new method on thousands of preparations from different tuberculous material: films of Koch-cultures, sputa from patients suffering from pulmonary tuberculosis, tuberculous cerebro-spinal fluids, abscesses, urines etc., and sections of organs. The result has in such cases been that the tubercle bacilli appeared prettily dark-blue coloured and — in comparison with those stained by the Ziehl-Neelsen — larger, with sharper outlines, more clearly standing out against the background (red or yellow), and more numerous, all this resulting in their being much easier to detect (important in routine-work).

With regard to tuberculous sputa it may be stated that all the other bacteria of the buccal flora took the counter-stain, but with one exception. Thus, it was possible, from time to time, to detect cells (ovoid, round, and branched) of a yeast-oidium-like fungus, the majority of which were counter-stained, but a good many had assumed the same dark-blue colour as the tubercle bacilli, proving that both organisms contain a substance to which Nachtblau possesses the same affinity. — It might be mentioned that this fungus fully corresponds to that which Reenstierna, in 1912 (studying it in *single cell* cultures), found able to produce acid-fast rods with the same morphological and tinctorial qualities as Koch's bacillus, observations confirmed and developed by Gullberg and by Hollström.

To judge from the publications (from Sweden and abroad), all research-workers, who have tried the Nachtblau method, seem to be of the same opinion as the writer regarding the said appearance of the tubercle bacilli when stained according to the new method and their being easier to detect. With regard to the question as to whether tubercle bacilli are really stained in larger numbers by Nachtblau

than according to Ziehl-Neelsen there are research-workers, e. g. Dissmann, who, after meticulous examinations, emphasize that the percentage of stained tubercle bacilli is higher when the Nachtblau method is used, while others, e. g. Kristenson, are of the opinion that the number of bacilli is equal, and that their appearance of being less numerous in the Ziehl-Neelsen preparations probably is due to the fact, that, especially in thick films, several of the germs are covered by the methylene-blue and, moreover, to an inferior combination of colours. At any rate, all research-workers conclude that the Nachtblau staining, from *practical* points of view, is definitely superior to the method of Ziehl-Neelsen which since its genesis, in 1883, has kept all other methods for staining tubercle bacilli on a certain distance. — As to some comparative examinations by the use of the fluorescence-microscope vide Chapt. VI.

Leprosy bacilli and other acid-fast micro-organisms (e. g. the bacilli in John's, Trautmann's, and Gellert's diseases, so-called saprophytes, as well as Mooser's "Oval Bodies") stained with Nachtblau, show, which was expected, acid-fastness. With regard to staining sections of lepromatous tissue the Nachtblau method is clearly superior to Ziehl-Neelsen's.

Apart from the aforesaid yeast-oidium-like fungus, often occurring in sputa and other material from tuberculous patients, also the common moulds (*Aspergillus*, *Penicillium*, and *Mucor*) and the *Actinomyces* take the Nachtblau stain, at least to a certain degree (specially in parts of young forms). But the writer has never met with these fungi on examining tuberculous material.

Several *scientific* findings have been obtained by the use of the Nachtblau method on material where the Ziehl-Neelsen had given negative results (in lupus vulgaris, Trautmann's and Trautmann's diseases, and leprosy). Cf. Chapters VI and VII.

Bibliography.

1. Andersson, Anna: On establishing the presence of tuberculosis bacilli by means of the fluorescence microscope. *Acta Med. Scand.*, 1943, Vol. CXV, p. 441.
2. Bertolini, G.: *Riv. ital. Igiene*, 1942, Vol. 2, p. 53. *Ref. Zbl. f. d. ges. Tbk.-Forschg.*, 1942—43, Bd. 55, p. 422.
3. Bosma, M. J.: Onderzoekingen omtrent de aetiologie der lepra. *Geneesk. Tijdschr. v. Nederl. Indië*, 1936, Afl. 34, Deel 72, p. 2163.
4. Dissmann, E.: Erfahrungen mit der Karbolnachtsblaufärbung der Tuberkelbazillen nach Hallberg. *Zbl. f. Bakt.*, 1943, Abt. I, Orig., Bd. 150, p. 268.
5. Ehrlich, P.: *Deutsch. Med. Wochenschr.*, 1882, Nr. 19, p. 269. — *Aus d. Verein f. Innere Med. zu Berlin. Sitzung 1. Mai 1882.*
6. Freundlich, H. and Neumann, W.: Zur Systematik der Farbstofflösungen. *Koll. Zeitschr.*, 1908, Bd. 3, p. 80.
7. Gellerstedt, N.: Discussion insertion. *Nord. Medicin*, 1940, Nr. 4, p. 164.
8. Gellerstedt, N.: On an isolated tuberculoid form of skin-granuloma, caused by atypical, acid-fast bacilli. *Acta Path. et Microbiol. Scand.*, 1944, Suppl. LIV.
9. Gellerstedt, N.: En tuberkuloid, isolerad hudaffektion, orsakad av atypiska syrafasta baciller. *Nord. Med.*, 1944, Nr. 23, p. 1604.
10. Grönwall, A. and Zetterberg, B.: Über die Technik der in vitro Untersuchung der auf Tuberkelbacillen wirksamen Chemotherapeutika und die Rolle der Paraaminobenzoesäure als "essentieller Metabolit". *Acta Med. Scand.*, 1946, Suppl. CLXX, p. 517.
11. Gullberg, E.: Some observations indicating the possibility of a relation of the bacillus of Koch to a yeast-like fungus (of *Oidium* type). — A posthumous paper. — *Acta Med. Scand.*, 1938, Vol. XCIV, p. 527.
12. Hallberg, V.: En ny metod för tuberkelbacillfärgning. — Meeting of the Swedish Assoc. of Pathol., Upsala, Nov. 4th, 1939. — *Nord. Med.*, 1940, Nr. 4, p. 164.
13. Hallberg, V.: A new method for staining tubercle bacilli. *Acta Med. Scand.*, 1941, Vol. CVIII, p. 12.
14. Hamperl, H.: Hallbergsche Färbemethode von Tuberkelbazillen. *Med. Klin.*, 1941, Nr. 20, p. 521.
15. Hedin, S. G.: Grundzüge d. physikal. Chemie in ihrer Beziehung z. Biologie, Wiesbaden 1915, p. 78.
16. Hedström, H.: Etiologiska undersökningar av s. k. hudtuberkulos hos nötkreatur. *Skand. Veterinärtidskr. för bakteriöl., patol. etc.*, 1941, p. 577.
17. Hellerström, S. and Hallberg, V.: Existe-il un lupus d'inoculation primaire? *Acta Dermat.-Venereol.*, 1943, Vol. XXIV, p. 298.
18. Hollström, E.: Some observations on the morphology of the tubercle micro-organism with special regard to its mycotic nature. *Acta Med. Scand.*, 1942, Vol. CXI, p. 303.

19. Hollström, E.: An investigation into a yeast-like fungus isolated from patients suffering from, or suspected of, pulmonary tuberculosis. *Acta Med. Scand.*, 1943, Suppl. CXLIV.
20. Hollström, E.: A fungus capable of producing acid-fast rods with special regard to its occurrence in Lymphogranulomatosis benigna (Schaumann's disease). *Acta Dermat.-Venereol.*, 1945, Vol. XXVI, p. 37.
21. Johne and Frothingham, Ein eigentümlicher Fall von Tuberkulose beim Rinde. *Deutsch. Zeitschr. f. Tiermed.*, 1895, Bd XXI, p. 438.
22. Kedrowski, W. I.: Ueber die Cultur des Lepraerregers. *Zeitschr. f. Hygiene etc.*, 1901, Bd. 37, p. 52.
23. Koch, R.: Die Aetiologie der Tuberculose. *Berlin. Klin. Wochenschr.*, 1882, Jahrg. 19, Nr. 15, p. 221. — Nach einem in der physiol. Gesellschaft zu Berlin am 24. März cr. gehaltenen Vortrage. —
24. Koch, R.: Über Tuberkulose. *Arch. f. Anat. u. Physiol.* 1882, *Physiol. Abt.* p. 190. — Aus d. Sitzung d. Berlin. *Physiol. Ges.* vom 24 März 1882. —
25. Koch, R.: Die Aetiologie der Tuberkulose. *Mitteil. a. d. Kais. Ges. Amte*, Berlin 1884, Bd. 2, p. 1.
26. Kristenson, A.: Några erfarenheter med färgning av tuberkelbaciller ad modum Hallberg. *Sv. Läkartidn.*, 1940, Nr. 20, p. 892.
27. Larsen, G.: Om 3 nyere metoder til påvisning av tuberkelbasiller. *Nord. Med.*, 1940, Nr. 44, p. 1874.
28. Moiser, B., Acid-fast oval bodies in cockroaches. *Internat. Journ. of Leprosy*, 1944, p. 97 and 112.
29. Neelsen, F.: Ein casuist. Beitr. z. Lehre v. d. Tuberkulose (Fussnote). *Cbl. f. d. Med. Wissensch.*, 1883, Jahrg. 21, p. 497.
30. Nyman, G. E., Linell, F., and Vahlne, G.: Om värdet av Nachtblaufärgning av tuberkelbaciller enligt Hallberg. *Nord. Med.*, 1944, Nr. 9, p. 398.
31. Quandt, J.: Erfahrungen mit der Karbolnachtblaufärbung der Tuberkelbazillen im Sputum und Gewebe. *Zbl. f. Bakt.*, 1944, *Abt. I Orig.*, Bd. 151, p. 348.
32. Reenstierna, J.: Ueber die Kultivierbarkeit und Morphologie des Lepra-Erregers und die Uebertragung der Lepra auf Affen. *Inaug.-Diss. Arch. Dermat. Syphil.*, 1913, Bd. CXVI, p. 480. — Vide also *Internat. Journ. of Leprosy*, 1938, Vol. 6, No. 1, p. 77.
33. Reenstierna, J.: Estudos sobre os microorganismos da lepra e da tuberculose. *Boletim Acad. Nac. Med.*, Rio de Janeiro, 1920, 91.º Anno, No. 20.
34. Reenstierna, J.: Sur la vie non acido-résistente du microorganisme de la tuberculose. *Arch. Inst. Pasteur de Tunis*, 1926, T. XV, p. 309.
35. Reenstierna, J.: The use of the Hallberg method for staining leprosy bacilli. Note in Hallberg's above paper (*Acta Med. Scand.*, 1941, Vol. CVIII, p. 15).
36. Reenstierna, J.: Leprans diagnostik. *Sv. Läkartidn.*, 1945, Nr. 7, p. 389.
37. Rindfleisch, E.: Tuberkelbacillen im Sputum. *Berlin. Klin. Wochenschr.*, 1883, Jahrg. 20, Nr. 12, p. 183.

38. Roulet, F.: Zur Nachtblaufärbung des Tuberkelbazillus nach V. Hallberg. Zbl. f. allg. Pathol. u. pathol. Anat., 1942/43, Bd. 80, p. 52.
39. Rubarth, S., and Agrell, I. M.: Tuberkelbacillfärgning ad modum Hallberg. Skand. Veterinärtidskr., 1941, Bd. 31, p. 641.
40. Schaumann, J.: On the nature of certain peculiar corpuscles present in the tissue of Lymphogranulomatosis benigna. Acta Med. Scand., 1941, p. 239.
41. Schaumann, H., and Hallberg, V.: Koch's bacilli manifested in the tissue of Lymphogranulomatosis benigna (Schaumann) by using Hallberg's staining method. Acta Med. Scand., 1941, Vol. CVII, p. 499.
42. Tegström, A.: A case of accidental laboratory tuberculous eye-infection. Acta Tuberculos. Scand., 1942, Vol. XVI, p. 330.
43. Traum, J., Case regards of lymphangitis in cattle caused by an acid-fast organism Journ. Amer. Vet. Med. Ass., 1916, Vol. 2, p. 254.
44. Törnell, E., Är lymphogranulomatosis benigna en svampsjukdom? Nord. Med., 1946, Nr. 38, p. 2149.
45. Törnell, E., Thresher's lung. A fungoid disease resembling tuberculosis or Morbus Schaumann. Acta Med. Scand., 1946, Vol. CXXV, p. 191.
46. Walker, E.: Some new aspects of the etiology and endemiology of leprosy. Journ. Preventive Medicine, 1929, Vol. 3, No. 3, p. 167.
47. Wihman, G., Weitere Versuche mit einer Anreicherungs-methode für Tuberkelbazillen in Sputum. II, Acta Tuberculos. Scand., 1944, Vol. XVIII, p. 235.
48. Ziehl, F.: Zur Färbung des Tuberkelbacillus. Deutsch. Med. Wochenschr., 1882, Nr. 33, p. 451.

PLATES I—IV
WITH EXPLANATIONS

The microphotographs have been taken by Dr. A. Frantzell
and the drawings made by Miss E. Öhrvall.

Explanation of Plates.¹

Plate I.

(Figs. 1, 2, 3, 5, and 6 are drawings; fig. 4 is a microphotograph.)

Fig. 1. Smear from a two months old glycerin bouillon culture of Koch's bacillus. Stain: Nachtblau-pyronin. Magnification, 1,000 diameters.

(Figs. 2—6. Smears from sputa of five patients suffering from pulmonary tuberculosis.)

Fig. 2. Only tubercle bacilli are seen. Stain: Nachtblau-pyronin. Magnification, 1,000 diameters.

Figs. 3 and 4. Besides Koch's bacilli there are seen fungus elements (ovoid, round, and pear-shaped cells, as well as mycelial branches) taking also dark-blue colour. A few of the mycelial branches are not very much thicker than the tubercle bacilli. Stain: Nachtblau-pyronin. Magnification, 1,000 diameters.

Fig. 5. Except three extremely small corpuscles and two tubercle bacilli we find an oval fungus cell from which issues a thread of the same thickness as the said bacilli. Stain: Simultaneous method. Magnification, 1,000 diameters.

Fig. 6. Tubercle bacilli and longer filaments of the same thickness as the former. Stain: Nachtblau-pyronin. Magnification, 1,000 diameters.

¹ As the German »Agfacolor Neu» film used for taking the microphotographs here reproduced is rather a recent invention, regarding which experience is somewhat incomplete, it has, several times, been impossible to avoid the occurrence of a certain light-blue, ground colouring that has nothing to do with the Nachtblau stain. This colouring is, undoubtedly, due to the extraordinary sensitivity of the film to variations of the quality of light.

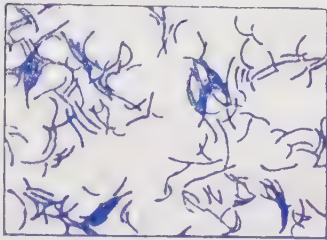


Fig. 1.

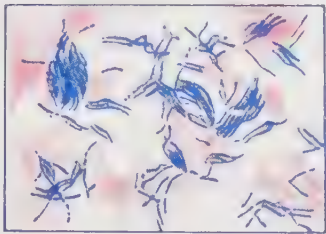


Fig. 2.

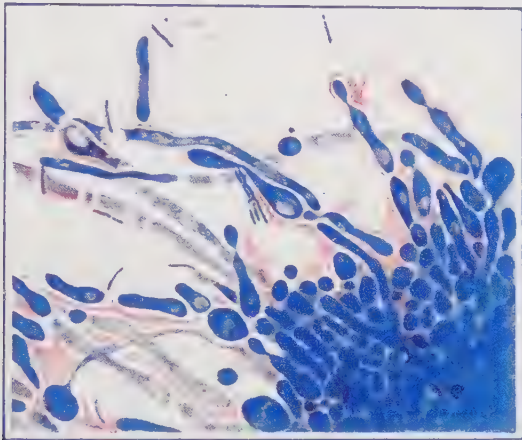


Fig. 3.

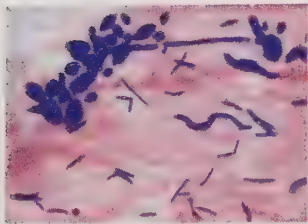


Fig. 4.

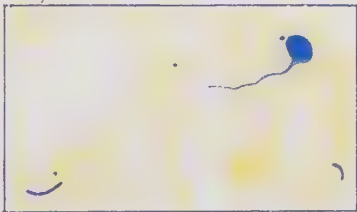


Fig. 5.

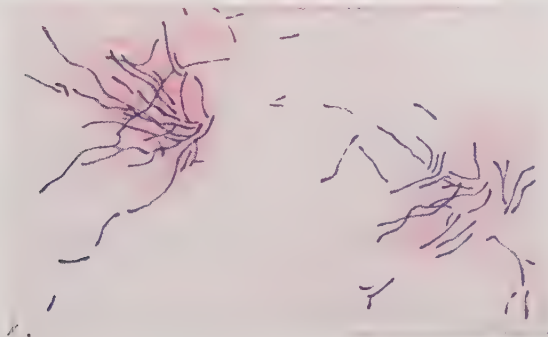


Fig. 6.

Plate II.

(Figs. 1, 2, 4, and 6—8 are drawings, fig. 3, 5, 9, and 10 microphotographs.)

Fig. 1. Section from a human tuberculous liver. Stain: Nachtblau-pyronin. Magnification, 1,000 diameters.

Fig. 2. Section from a human tuberculous spleen. Stain: Simultaneous method. Magnification, 1,000 diameters.

Fig. 3. Smear from the cerebro-spinal fluid (a case of tuberculous meningitis) showing a pear-shaped fungus cell. — In another field of vision Koch-bacilli were found. — Stain: Nachtblau and neutral-red. Magnification, 1,000 diameters.

Fig. 4. Smear from inguinal glandular pus of a guinea-pig which six weeks earlier had been inoculated in the corresponding leg with tuberculous sputum. Numerous small granules, partly arranged as streptococci, and two agglutinated Koch-bacilli are seen. Stain: Simultaneous procedure. Magnification, 1,000 diameters.

Fig. 5 (reproduced from Schaumann's paper of 1941). Section from a lymphatic gland invaded by L. B. In the centre there is seen, among the epithelioid red-stained cells, a round, double-contoured, blue corpuscle (fungus cell). Stain: Nachtblau-pyronin. Magnification, 1,000 diameters.

Figs. 6—8 (reproduced from Schaumann and Hallberg's paper of 1941). Section from a lymphatic L. B. gland. Blue-coloured fungus cells and rods of Koch-bacillus-type. Stain: Nachtblau-pyronin. Magnification, 1,000 diameters.

Figs. 9 and 10. Smears from a strain (single cell culture in glycerin bouillon) of the aforesaid yeast-oidium-like fungus isolated from a patient suffering from pulmonary tuberculosis. In fig. 9 (after four days) the majority of the fungus cells are acid-fast, in fig. 10 (after six weeks) non-acid-fast. — Note the large faintly rose-coloured formation (a swollen fungus cell) in the centre of fig. 9. — Stain: Nachtblau-pyronin. Magnification, 1,000 diameters.

The last two microphotographs were placed at the writer's disposal by the courtesy of Dr. E. Hollström, Lecturer of Hygiene and Bacteriology, at Uppsala.

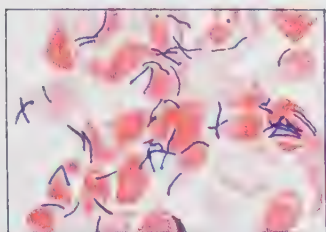


Fig. 1.

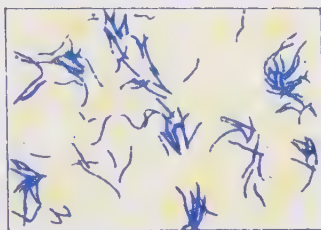


Fig. 2.



Fig. 3.

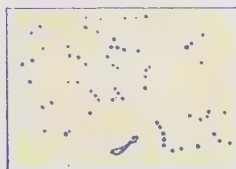


Fig. 4.



Fig. 5.



Fig. 6.



Fig. 7.



Fig. 8.

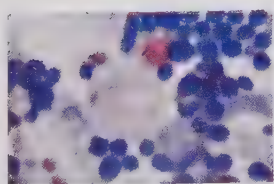


Fig. 9.

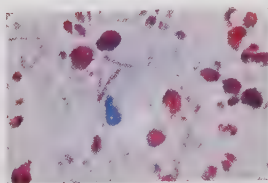


Fig. 10.

Plate III.

Sections of a leproma originating from a Swedish patient. Fig. 1. Stain: Nachtblau-pyronin. Fig. 2. Simultaneous method. Magnification, 1,000 diameters.

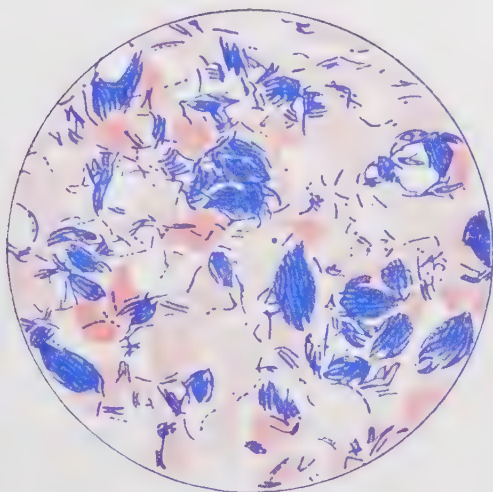


Fig. 1.

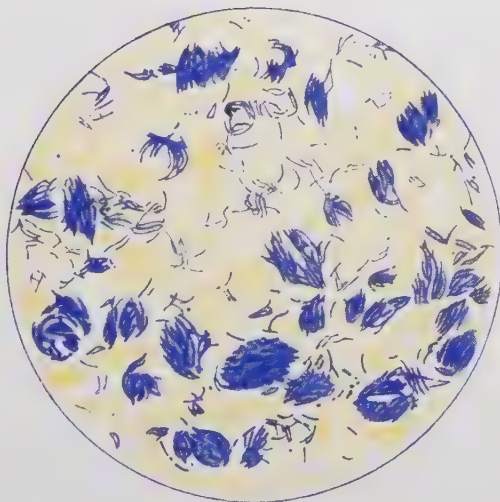


Fig. 2.

Plate IV.

(Figs. 1 and 3 are microphotographs, figs. 2 and 4—8 drawings.)

Fig. 1 (reproduced from Reenstierna's note to the writer's aforesaid paper of 1941). Direct film of blood taken, in 1939, from the arm vein of a second Swedish leper. Blue-coloured, ovoid fungus cells. — In some films Hansen's bacilli could be detected, sporadically, both by using the Nachtblau and the Ziehl-Neelsen method. — Stain: Nachtblau and neutral-red. Magnification, 1,000 diameters.

Fig. 2. Thick film of blood taken, in 1942, from the arm vein of a third Swedish leper. A spindle-shaped fungus cell, blue-coloured with a touch of red, is seen. Stain: Nachtblau-pyronin. Magnification, 1,000 diameters.

(Figs. 3 and 4 reproduced from Reenstierna's aforesaid note.)

Fig. 3. Nasal smear from an Indian leper. A group of about twenty-five ovoid and pear-shaped fungus cells are seen and at least one Hansen-bacillus (in the left upper corner). Stain: Nachtblau and neutral-red. Magnification, 1,000 diameters. — The film provided, in 1938, by the courtesy of Dr. R. G. Cochrane, Medical Superintendent of Lady Willingdon Leper Settlement, Chingleput, Madras, South India.

Fig. 4. Smear from the culture in a fluid medium (glycerin bouillon, ascitic fluid, glucose, and lumps of human brain) of a micro-organism isolated by Reenstierna, in 1924, from the cerebro-spinal fluid of a fourth Swedish leper. This film, which previously was stained by Ziehl-Neelsen, is stained afterwards with Nachtblau and neutral-red, but decolorised by 96 per cent. alcohol instead of 70 per cent. A number of blue corpuscles are seen in between the specimens of the streptococcus-like, red-stained micro-organism. The writer is unable to say whether this blue staining implies a certain degree of acid-fastness. It should be noted, furthermore, that the micro-organism in question, when stained by Ziehl-Neelsen, took the methylene-blue colour, sometimes, however, showing reddish elements. Magnification, 1,000 diameters.

Fig. 5. Intestinal section from a case of Johne's disease. Stain: Nachtblau-pyronin. Magnification, 1,000 diameters.

Fig. 6. Section of a skin lesion from a case of the disease, in man, described by Gellerstedt. Stain: Nachtblau-pyronin. Magnification, 1,000 diameters.

The preparations were placed at the writer's disposal by the courtesy of Dr. N. Gellerstedt, Associate Professor of Pathology, at Uppsala.

Figs. 7 and 8. Section of a fresh skin lesion from a case of the disease, in cattle, described by Traum. In fig. 7 are seen groups of acid-fast, short bacilli closely resembling those illustrated in fig. 6. Some of the agglomerations are the size and have the outlines of fungus cells. Fig. 8 shows five fungus cells together with two rods. Stain: Simultaneous method. Magnification, 1,000 diameters.

The skin nodule from which the sections were cut was placed at the writer's disposal by the courtesy of Dr. H. Hedström, Associate Professor of Bacteriology at the State Veterinary Medical Institute, in Stockholm.

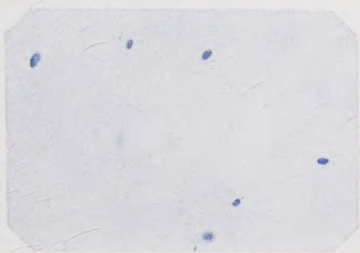


Fig. 1.

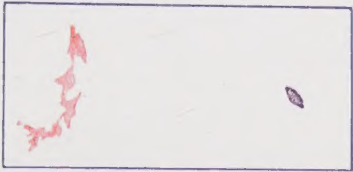


Fig. 2.

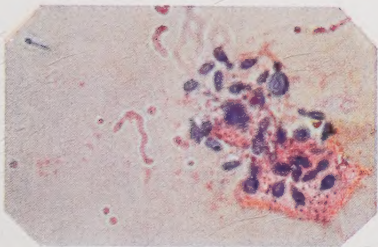


Fig. 3.



Fig. 4.

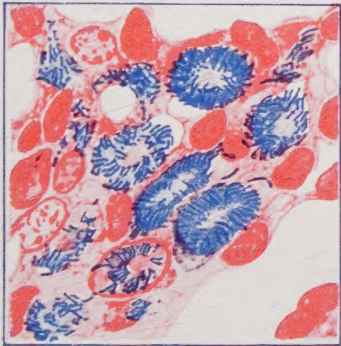


Fig. 5.

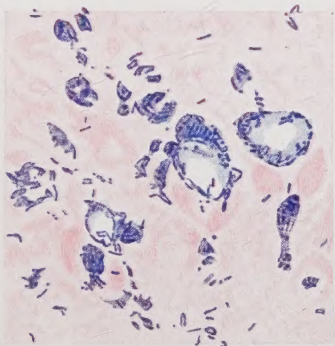


Fig. 6.

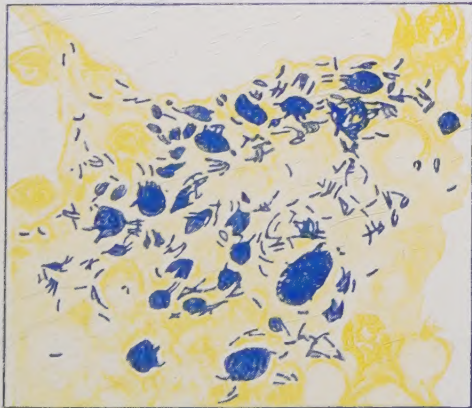


Fig. 7.



Fig. 8.

